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In vitro conservation of tropical plant germplasm – a review

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Summary

In vitro medium term conservation of tropical plant germplasm is used routinely in many laboratories. Growth reduction is achieved by modifying various parameters, such as temperature, culture medium, gaseous environment. For long term conservation, cryopreservation (i.e. storage in liquid nitrogen, – 196° C) is the only current method available. Each successive step of the process requires precise conditions which have to be defined for each material. Cryopreservation protocols have been set up for more than 40 tropical species. Results obtained with various culture systems such as cell suspensions, protoplasts, calluses, meristems and embryos are discussed. The first example of the large scale application of cryopreservation (oil palm somatic embryos) is presented.

1. Introduction

As regards seed preservation possibilities, plant species have been divided into 2 categories (Roberts, 1973):
– orthodox seeds, which can withstand dehydration to 5% or less (dry weight basis) without damage. When dry, the viability of these seeds can be prolonged by keeping them at the lowest temperature and moisture possible.
– recalcitrant seeds, which are high in moisture and are unable to withstand much desiccation. They are predominantly seeds from tropical or subtropical species. They can be stored only in wet medium in order to avoid dehydration injury and in relatively warm conditions because chilling injury is very common among these species. They remain viable only for a short time (weeks or months), even if kept in the required moisture conditions. This group comprizes many crop species of great economic importance such as oil palm, coconut, cacao, coffee. Moreover, there are practical problems in applying long-term seed storage to most long-live forest trees, including gymnosperms and angiosperms, since their juvenile period is very long and they do not produce seeds for several years.

The conservation of plants which are vegetatively propagated, such as cassava, potato, yam, poses also considerable problems.

In situ conservation has been made almost impossible due to the disappearance of large wild areas. Conservation *ex situ* is very difficult to carry out due to the following problems: an adequate sample has to be taken for the conservation of genetic diversity. It varies from 20 to 30 plants for a single population, to several hundreds for gene pool conservation and to 5,000–20,000 plants, depending on the species, for the maintenance of heterozygosity. Thus, land space requirement is very important, particularly in the case of forest trees, which are often very large, whereas land availability drastically decreases. Moreover, in the

case of heterozygous species, it is necessary to preserve a larger sample to maintain as much as possible of the genetic variation within a population. Labour costs and trained personnel requirements are very important. Moreover, plant material in natural conditions remains exposed to natural disasters, pests and pathogens and is submitted to threats from changing government policies and urban development. Finally, for many species, we do not possess even the rudiments of knowledge of the biology of the species.

During the last years, *in vitro* culture techniques have been extensively developed and applied to more than 1,000 species, including many tropical species. The use of *in vitro* tissue culture techniques can be of great interest for germplasm collection, storage and multiplication of recalcitrant and vegetatively propagated species. Tissue culture systems present advantages which are listed below:

(1) very high multiplication rates
(2) aseptic system:
  - free from fungi, bacteria, viruses (after thermotherapy and indexation) and insect pests
  - production of pathogen-free stocks
(3) reduction of space requirements
(4) genetic erosion reduced to zero under optimal storage conditions
(5) reduction of the expenses in labour costs

Moreover, tissue culture systems greatly facilitate the international exchange of germplasm. Indeed, the size of the samples is drastically reduced and they can be shipped in sterile conditions.

However, the *in vitro* storage of large quantities of material induces various problems: laboratory management of plant material which needs to be regularly subcultured, risks of genetic variation, which increase with *in vitro* storage duration, and can lead to the loss of trueness to type.

The methods employed are different, depending on the storage duration requested. For short and medium term storage, the aim is to reduce the growth and to increase the intervals between subcultures. This is achieved by modifying the culture conditions, mainly by lowering the culture temperature.

For long term storage, cryopreservation, i.e. storage at very low temperatures, usually that of liquid nitrogen (−196° C), is the only current method. At this temperature, all cellular divisions and metabolic events are stopped. The plant material can be stored without alteration or modification, for a theoretically unlimited period of time. Moreover, the cultures are stored in a small volume, protected from contamination, requiring a very limited maintenance.

In this paper, the principal techniques employed for the short and medium term storage of tropical plants are described firstly. As concerns cryopreservation, the methodology of a cryopreservation protocol is analysed and its current applications to various culture systems (cell suspensions, protoplasts, calluses, meristems, embryos) are discussed. The example of its large scale application to oil palm somatic embryos is finally detailed.

2. Short and medium term storage

2.1 Principal factors

2.1.1 Temperature

Growth reduction is generally achieved by lowering the culture temperature. In several cases, the cultures are maintained at standard temperature. However, satisfactory storage durations are obtained only with slow growing species. For example, *Coffea arabica* plantlets conserved at 27° C can be subcultured every year but *Coffea racemosa* plantlets have to be transferred every 6 month (Bertrand-Desbrunais & Charrier, 1990).

Tropical plant species are generally cold sensitive. The physiological damages induced by cold are commonly referred to as chilling injury (Lyons, 1973; Graham & Patterson, 1982). They include various changes in the metabolism, protein content, composition and functioning of the membranes. These disorders increase with the degree of chilling, however they are generally reversible following short term exposure to low temperature.

The storage temperature depends on the cold sensitivity of the species. Kiwi shoots are conserved at 8° C (Monette, 1986), *Colocasia esculenta* cultures tolerate 3 years of storage at 9° C (Zandvoort & Staritsky, 1986). On the contrary, cassava plantlets

have to be stored at temperatures higher than 20° C (Roca et al., 1984). Oil palm plantlets and somatic embryos are not able to resist a relatively short exposure to temperatures lower than 18° C (Corbineau et al., 1990).

Staritsky et al. (1985) increase the storage duration of *Colocasia esculenta* shoots at + 3° C by exposing them for 48 hours to 18–22° C every 15 days. This treatment appeared to induce a partial reversion of physiological disorders induced by cold. The same process proved to be successful for potato shoot preservation (Westcott, 1981a).

A reduction in light intensity or a complete suppression is often used in combination with temperature reduction. Shoot cultures of banana are easily stored at 15° C under a 1000 lux light intensity (Banerjee & De Langhe, 1985). The need for light is not universal and varies from one species to another.

2.1.2 Culture medium

Various alterations to the culture medium can be made:

– lowering the content in mineral elements and/or sugar. Kartha et al. (1981) could preserve *Coffea arabica* plantlets for 2 years on a medium devoid of sugar and with only half of the mineral solution of the standard medium.

– addition of cryoprotective substances or substances with osmotic properties. The addition of mannitol reduces significantly the growth of *Colocasia esculenta* and *Xanthosoma brasiliense* shoots (Staritsky et al., 1985). High sucrose concentrations allow the conservation at low temperature of protocorms of *Cymbidium* spp. and *Dendrobium chrysanthum* and *D. ochreatum* (Homes et al., 1982; Tandon & Sharma, 1986). The growth of potato shoots at 25° C is reduced by the addition of 4% mannitol (Espinoza et al., 1986). The same cultures can withstand storage at a lower temperature, thus extending the possible storage duration, if 3% sucrose and 4% mannitol are added to the culture medium. However, cassava shoots deteriorate in presence of mannitol, even at 0.1% and with a storage temperature lower than 20° C (Roca et al., 1982).

– growth retardants can be added: Westcott (1981b) and Roca et al. (1982) use absisic acid (ABA) in order to reduce the growth of shoots of potato. However, these authors indicate that ABA is detrimental to some varieties.

– finally, other substances are sometimes added. Roca et al. (1984) observe that the addition of activated charcoal has positive effects on the storage of cassava shoots: it reduces defoliation, decreases and nearly halves shoot growth for one genotype, limits chlorophyll degradation and browning of roots.

2.1.3 Physiological stage of the explants

The type of explants as well as their physiological stage are very important. There is a minimal size for the explants. The presence of a root system increases the survival capacities, as observed notably by Kartha et al. (1981) with *Coffea arabica* plantlets. Microtubers instead of shoot cultures can be successfully employed for the storage of potato germplasm (Kwiatkowski et al., 1988). The duration between the last transfer and the moment when the cultures are placed in storage conditions can be of great importance: it is sometimes better to store the cultures immediately after the transfer, thus avoiding the occurrence of necrosis and production of phenolic compounds.

2.1.4 Culture vessel

Finally, the type of culture vessel can play a very important role. Test tubes or plastic boxes containing 10 to 20 ml of medium are routinely used, which allows an increase in the number of replicates of each genotype and to limit the incidence of contaminations. Roca et al. (1984) indicate that, when storing cassava plantlets in 50 × 140 mm bottles instead of 25 × 150 mm test tubes, the rate of shoot elongation in larger vessels almost doubled; however, leaf fall diminished and culture viability greatly increased. In addition, leaves and roots remained healthier in the large than in the small vessels. The use of sterile, heat sealable polypropylene bags has been mentioned recently (Reed, 1990). They may present advantages, such as improvement in gaseous exchange, reduction of the risks of contamination, reduction of the occupied surface in the culture rooms.

2.1.5 Modifications of gaseous environment

Growth reduction can be achieved by lowering the oxygen level. Several methods exist in order to decrease the quantity of oxygen available for the tissues. The simplest is to cover the tissues with mineral oil. This technique was firstly developed by Caplin (1959) with carrot calluses, and successfully employed recently by Augereau et al. (1986) with *Catharanthus* calluses and Moriguchi et al. (1988) with grape calluses for their medium term conservation.

Several attempts have been made for storing organized structures using this technique (Chatti-Dridi, 1988; Jouve et al., 1991). Indeed, growth reduction is obtained but vitrification is oftenly observed during storage. Moreover, when returning to standard conditions, regrowth is very slow and partial or complete necrosis of the explants is sometimes observed.

Another method consists in lowering the oxygen partial pressure using controlled atmospheres or decreasing the atmospheric pressure of the culture chamber. Tobacco and chrysanthemum plantlets could be stored for 6 weeks under 1.3% oxygen, without impairing their further development (Bridgen & Staby, 1981). This technique was reemployed recently (Engelmann, 1990b) for the storage of oil palm somatic embryos. After 4 months in an atmosphere containing 1% O_2 reproliferation could be obtained very rapidly from the whole culture, whereas control embryos cultivated in standard conditions were severely damaged. This method seems particularly attractive for the storage of tropical species, due to their cold sensitivity. Indeed, growth reduction can be achieved without reducing the culture temperature.

2.1.6 Encapsulation

This technique is now commonly used in the 'synthetic seeds' technology by coating somatic embryos in alginate beads. Some preliminary conservation experiments have been carried out recently using this technique. Mulberry buds and sandalwood somatic embryos encapsulated in alginate could be stored for 45 days at + 4° C and resume growth after the storage period (Bapat et al., 1987; Bapat & Rao, 1988). The storage duration was extended recently to 4 months with *Podophyllum hexandrum* somatic embryos (Arumugam & Bhojwani, 1990). This technique may be very promissing in a near future for conservation purposes. Indeed, the protection provided to the plant material by encapsulation could increase its resistance to dehydration and low temperature, thus opening new possibilities for medium term storage.

2.1.7 Desiccation

Several attempts have been made using partial desiccation of plant material. Nitzsche (1980) could store desiccated carrot calluses for one year and revive them. McKersie et al. (1990) could dehydrate *Medicago sativa* somatic embryos down to 15% water and store them for 8 months at room temperature. These authors indicate that a pretreatment with ABA could increase the dehydration tolerance, thus improving the conservation possibilities.

2.1.8 Stability of stored plant material

If medium term storage of organized structures appears to be safer when considering trueness to type, it is not the case for the storage of cell lines or calluses. Indeed, several papers mention a rapid decrease in growth rate or/and biosynthesis capacities (Seitz, 1987). Moreover, even with organized material, such as shoot cultures, prolonged storage in more or less detrimental conditions may lead to the selection of particular genotypes, thus leading to the loss of a part of the germplasm stored.

2.1.9 Conclusion

Conventional medium term storage techniques are now routinely employed in many laboratories and international/regional centers (e.g. CIAT, CIP, CATIE). However, the management of large collections, even if the intervals between transfers are greatly extended, poses considerable problems (Roca et al., 1989). Thus, additional techniques, which suppress almost completely the needs for material maintenance, have to be sought. Cryopreservation is an obvious candidate.

3. Cryopreservation

3.1 Methodology

A cryopreservation process comprizes the following successive steps which have to be defined for every species: choice of material, pretreatment, freezing, storage, thawing, post-treatment.

3.1.1 Choice of material

As a general rule, material will be chosen as young and as meristematic as possible. Indeed, cells in this type of material are the most likely to withstand freezing: they are small, contain only a few vacuoles, i.e. only a small amount of water, their cytoplasm is dense, and their nucleo-cytoplasmic balance is high.

The material can be sampled from *in vivo* or *in vitro* plants. *In vitro* material is generally preferable, since the explants are already miniaturised, free of superficial contamination and may also be pathogen free.

The physiological stage of the material is very important. In the case of cell suspensions, material during the exponential growth stage is most likely to be able to successfully withstand freezing (Withers, 1985). With carnation meristems, survival decreases progressively with their rank on the shoot axis, starting from the terminal meristem (Dereuddre et al., 1989).

It is sometimes necessary to set up a special culture medium in order to obtain starting material in sufficient quantities. This is the case with oil palm somatic embryos (Engelmann & Dereuddre, 1988a); only a special type of embryos, shiny white, finger-like shaped, which are often grouped into clumps, are likely to withstand freezing. These particular embryoids are very rarely observed under standard culture conditions. Their frequency is increased by a two month culture on a medium containing 0.3 M sucrose instead of 0.1 M, as in the standard culture medium.

Recently, Harding et al. (1991) indicated that a long-term period of tissue culture before cryopreservation significantly reduced the ability of potato shoot-tips to survive freezing.

3.1.2 Pretreatment

The pretreatment corresponds to a culture of the material for a certain period of time (several minutes to a few days) under conditions which prepare it to the freezing process. It is carried out using various cryoprotective substances like sucrose, sorbitol, mannitol, dimethylsulfoxide, polyethylene glycol, which differ greatly one from the other by their molecular weight and their structure. The exact mode of action of these substances is unclear: they have an osmotic role and act thus by dehydrating the cells but they may act also by protecting membranes and enzymatic binding sites from freezing injury. They are sometimes classified in penetrating and non-penetrating compounds, the former having both above cited effects, the latter acting only as osmotica. As concerns the dehydration effect of cryoprotective compounds, the exact water content of cells can be determined in some cases by using Nuclear Magnetic Resonance.

For every species, one will have to determine the choice of cryoprotectants and their concentration as well as the duration of the pretreatment. In some cases, the pretreatment will have to be adapted to different clones or varieties of the same crop.

3.1.3 Freezing

Different types of freezing processes can be carried out: ultra-rapid, rapid, or slow freezing. In the latter case, a programmable freezing apparatus will be needed in order to obtain precise and reproducible freezing conditions.

At the cellular level, the different freezing processes described above correspond to different mechanisms as regards water fluxes and ice crystallization: during slow freezing, ice crystallization occurs firstly in the external medium. The water flows out of the cells to the external ice. The cells will have to be sufficiently dehydrated so as crystallization of the residual water will cause no damage but not too dehydrated in order to avoid toxicity due to the concentration of the internal solutes, which increases with dehydration. During rapid freezing, intracellular ice crystallizes in microcrystals of a size which is harmless to the integrity of the cell components.

For every material, the following criteria need to be determined:
– freezing rate: it can be very precise, as in the case of strawberry meristems (Kartha et al., 1980), or fall within a much broader range, as in the case of oil palm somatic embryos (Engelmann & Dereuddre, 1988b).
– starting and prefreezing temperature: i.e. the temperatures of beginning and end of programmed freezing. These parameters are oftenly very important: in the case of cassava meristems, a prefreezing temperature of $-20°$ C ensures 91% of survival; only 3.3% are observed if the controlled freezing stops at $-40°$ C (Kartha et al., 1982).

3.1.4 Storage
The maximal storage duration is theoretically unlimited, provided that the samples are permanently kept at or near the temperature of liquid nitrogen. The material does, however, remain exposed to natural radiation. It was calculated with animal cells that the level of mutations caused by natural radiations during storage may reach an irreparable level after thawing of the stored material only after extended periods of time (Ashwood-Smith & Friedmann, 1979).

3.1.5 Thawing
In the majority of the cases, thawing is carried out rapidly by immersing the cryotubes containing the samples in a water-bath thermostated at around $+40°$ C. The aim is to avoid fusion during thawing of the ice microcrystals formed during freezing. Fusion could form larger crystals of a size which would damage cellular integrity. However, slow thawing is sometimes necessary (Withers, 1979; Marin et al., 1990).

3.1.6 Post-treatment
Post-treatment consists of culturing the material under conditions that ensure its optimal recovery in the best possible conditions. Cryoprotective substances are progressively eliminated by rinsing, dilution or diffusion, for they are toxic if kept too long in contact with the material.

It is sometimes necessary to attenuate the osmotic shock caused by an immediate transfer onto a medium with low osmotic potential by successive transfers of the material to progressively less concentrated media (Engelmann et al., 1985). In some cases, the nature of the medium must be changed (solid versus liquid), in order to improve the regrowth. With cell suspensions, a transitory culture phase on solid medium is commonly used before returning to liquid conditions.

Recovery can eventually take place in the dark, in order to avoid photooxidation phenomena which can be harmful to the material (Benson et al., 1989).

Finally, the composition of the culture medium can be transitorily modified (mineral composition, hormonal content).

3.1.7 Viability assessment
In many cases, regrowth is very slow. The only definitive assessment of viability is regrowth after thawing. However, it is very important to know as soon as possible if material is living after freezing. Two main tests exist in order to measure the viability of the material, which can be applied very rapidly after thawing. These tests are:
– FDA (fluorescein diacetate): FDA is absorbed by the living cells and transformed into fluorescein, whose fluorescence is induced by UV irradiation. This test is quantitative in that the percentage of fluorescing cells can be counted (Widholm, 1977).
– TTC (2, 3, 5 – triphenyl tetrazolium chloride): TTC is reduced to formazan, colored red, by respiration in the mitochondria of the living cells. This test is quantitative for cell suspensions but is only qualitative for large tissues and organs (Steponkus & Lanphear, 1967).

The major disadvantage of viability tests is that they are destructive. Moreover, as observed recently by Panis et al. (1990), the FDA test is very precise for estimating viability, but gives no informations on the capacity of the cells to proliferate. Non destructive methods for estimating the viability of the material are sought, such as chromatographic analysis of volatile hydrocarbon production (ethylene, ethane) by cryopreserved tissues (Benson & Withers, 1987).

3.2 Results

3.2.1 Various types of cultures

Today, cryopreservation has been applied to more than 70 species (Kartha, 1985; Dereuddre & Engelmann, 1987). Table 1 presents the list of the species of tropical origin which have been frozen as cell suspensions, calluses, protoplasts, meristems and embryos.

3.2.1.1 Cell suspensions

For cell suspensions, routine techniques adapted to a large number of species have been proposed for several years (Withers & King, 1980; Withers, 1985). As far as the setting up of particular conditions for the successive phases of a cryopreservation process are concerned, the following remarks can be made. The cells must be used during their exponential growth period (Withers & Street, 1977; Kartha et al., 1982). Before the application of the cryoprotective compounds, a pregrowth period of several hours or days in the presence of compounds with osmotic properties is sometimes necessary (Maddox et al., 1982–83; Seitz et al., 1983; Seitz & Reinhard, 1987). For cryoprotection, various substances are employed, individually or in binary or ternary mixtures. Sucrose (7 to 25%) is used with *Panax ginseng* cell suspensions (Butenko et al., 1984), DMSO (5%) with rice cells (Cella et al., 1982). A mixture of cryoprotectants is oftenly more efficient than only one component at the same total osmolarity. A mixture of polyethylene glycol, glucose and DMSO (10%, 8%, 10% respectively) is used for sugarcane cell suspensions (Finkle & Ulrich, 1982), DMSO and sorbitol in the case of *Catharanthus roseus* (Chen et al., 1984). For the freezing procedure, slow freezing (0.1 to 1° C · min^{-1}) is routinely used. Increasing the cooling rate generally leads to a decrease in viability, as observed by Chen et al. (1984). The prefreezing temperature usually falls between − 30 and − 40° C. In the case of *Catharantus* cells, − 40° C appears to be optimal (Kartha et al., 1982).

Rapid thawing is usually employed using a water bath thermostated at + 30/40° C. Increasing the temperature of the water bath to + 60° C or + 80° C can lead to an improvement of the results (Reuff et al., 1988). The same authors mention the utilization of a microwave oven for a more homogeneous thawing, which gave very good results.

As regards post-thaw treatments, cells can eventually be washed in order to remove the cryoprotectants (Butenko et al., 1984). However, this treatment is in the majority of the cases deleterious to the survival of the cells due to the osmotic shock created. To avoid this, the cryoprotective substances can be removed slowly by means of diffusion. Culture for a period on semisolid medium, generally for one or two weeks, is required for recovery before the cells are transferred again to normal culture conditions. The regrowth medium can be transitorily modified by incorporating compounds with osmotical properties, so as to reduce osmotic shock (Maddox et al., 1982–83). The mineral composition of the medium can be altered: in the case of rice cells, Kuriyama et al. (1989) showed that ammonium ions had an inhibitory effect on the regrowth of cryopreserved rice cells. Their suppression during the first subcultures led to an improvement of regrowth. In the case of *Lavandula vera* cells, the addition of activated charcoal to the recovery medium led to a significant improvement in survival (Kuriyama et al., 1990).

3.2.1.2 Protoplasts

For protoplast cryopreservation, the conditions of the successive steps are comparable to that developed for cell suspensions, but for the post-treatment: the protoplasts are resuspended immediately in liquid medium, and the cryoprotective medium is progressively diluted (Takeuchi et al., 1982).

3.2.1.3 Calluses

For callus cryopreservation, actively growing cultures are needed. They are submitted to a pretreatment with cryoprotective mixtures such as polyethylene glycol, glucose and DMSO for rice and date palm calluses (Finkle et al., 1982), or DMSO and glucose (Ling et al., 1987) with sugarcane calluses. Freezing is usually carried out slowly (freezing rate of 1° C · min^{-1}) to − 23° C (date palm) or

Table 1. List of tropical plant species cryopreserved as cell suspensions (a) calluses, (b) protoplasts, (c) meristems, (d) somatic, (e) pollinic, (f) and zygotic, (g) embryos

(a) cell suspensions	
Berberis dictyophilla	Withers, 1985
Berberis wilsoniae	Reuff, 1987
Brunifelsia dentifolia	Pence, 1990
Capsicum annuum	Withers & Street, 1977
Catharanthus roseus	Kartha et al., 1982
	Chen et al., 1984
	Withers, 1985
Corydallis sempervirens	Withers, 1985
Dioscorea deltoidea	Butenko et al., 1984
Glycine max	Bajaj, 1976
	Weber et al., 1983
Hyosciamus muticus	Withers, 1985
Musa	Panis et al., 1990
Myrtillocactus geometrizans	Haffner, 1985
Nicotiana plombaginifolia	Maddox et al., 1982–83
Nicotiana sylvestris	Maddox et al., 1982–83
Nicotiana tabacum	Withers, 1985
	Bajaj, 1976
	Hauptman & Widholm, 1982
Oryza sativa	Sala et al., 1979
	Finkle & Ulrich, 1982
	Ulrich et al., 1984
Panax ginseng	Butenko et al., 1984
	Chen et al., 1984
	Seitz & Reinhardt, 1987
Rhazia orientalis	Withers, 1985
Rhazia stricta	Withers, 1985
Saccharum officinalis	Finkle & Ulrich, 1979, 1982
	Gnanapragasam & Vasil, 1990
Solanum melongena	Withers, 1985
Sorghum bicolor	Withers & King, 1980
Tabernaemontana divaricata	Schrijnemakers et al., 1990
Vinca minor	Caruso et al., 1987
Zea mays	Withers & King, 1980
	Shillito et al., 1989
(b) callus	
Gossypium arboreum	Bajaj, 1982
Oryza sativa	Finkle et al., 1982
	Ulrich et al., 1984
Phoenix dactylifera	Tisserat et al., 1981
	Finkle et al., 1982
Saccharum spp.	Ulrich et al., 1979
	Ling et al., 1987
(c) protoplasts	
Glycine max	Takeuchi et al., 1982
	Weber et al., 1983
Nicotiana tabacum	Bajaj, 1988
Oryza x Pisum	Bajaj, 1983a
Zea mays	Withers, 1980
(d) meristems	
Arachis hypogeaea	Bajaj, 1979
Cicer arietinum	Bajaj, 1979
Lycopersicon esculentum	Grout et al., 1978
Manihot esculenta	Bajaj, 1977a, 1983b, 1985
	Kartha et al., 1982
Phoenix dactylifera	Bagniol et al., 1990
Solanum tuberosum	Towill, 1981
Solanum goniocalix	Grout & Henshaw, 1978
Solanum tuberosum	Standke, 1978
	Bajaj, 1985
	Benson et al., 1984, 1989
Xanthosoma	Zandvoort, 1987
Vanda hookeriana	Kadzimin, 1988
(e) somatic embryos	
Citrus sinensis	Marin & Duran-Vila, 1988
Coffea arabica	Bertrand-Desbrunais et al., 1988
Elaeis guineensis	Engelmann et al., 1985
	Engelmann & Duval, 1986
	Engelmann & Dereuddre, 1988b
Manihot esculenta	Sudarmonowati & Henshaw, 1990
Xanthosoma	Zandvoort, 1987
(f) pollen embryos	
Arachis hypogea	Bajaj, 1983c
Arachis villosa	Bajaj, 1983c
Citrus spp.	Bajaj, 1984
Gossypium arboreum	Bajaj, 1982
Nicotiana tabacum	Bajaj, 1977b, 1978
Oryza sativa	Coulibaly & Demarly, 1979
	Bajaj, 1981
(g) zygotic embryos	
Carva	Pence & Dresser, 1988
Camellia sinensis	Chaudury et al., 1991
Cocos nucifera	Bajaj, 1984
	Chin et al., 1989
Elaeis guineensis	Grout et al., 1983
Hevea brasiliensis	Normah et al., 1986
Howea fosteriana	Chin et al., 1988
Manihot esculenta	Marin et al., 1990
Musa	Mora et al., 1991
Veitchia merrillii	Chin et al., 1988
Zea mays	Delvallée, 1987
	de Boucault & Cambecedes, 1988

– 40° C (sugarcane). In the case of sugarcane, survival of cryopreserved material is obtained only if the samples are held for two hours at the terminal prefreezing temperature. Thawing is carried out rapidly and the calluses are rinsed with a simplified liquid medium containing 3% sucrose before being transferred onto standard semi-solid medium (Finkle et al., 1982). These authors underline the importance of the temperature at which the cryoprotective substances are added and removed: survival is obtained only when these operations are carried out at 0° C. Reproliferation of sugarcane callus is enhanced when it is performed in the dark (Ling et al., 1987).

In the case of cryopreservation of *Picea abies* callus, better regrowth is observed with frozen material, compared with controls (Bercetche et al., 1990). This appears to be due to the selection between different cell types imposed by cryopreservation: all the differentiated cells of the callus are killed during the freeze-thaw cycle and all the meristematic cells remain viable.

3.2.1.4 Meristems

In the case of meristems, the aim is to preserve the whole structure, which is of macroscopic size and to obtain its direct regrowth without adventive organogenesis. With potato and date palm meristems, survival is improved if the meristems are placed on standard medium for 1 to 3 days before any contact with cryoprotective substance, in order to recover from dissection and/or reinitiate growth (Benson et al., 1989; Bagniol et al., 1990). Pregrowth in presence of cryoprotective substances is frequently necessary (Kartha, 1982; Kartha et al., 1982). For the freezing procedure, there is no general rule: ultra rapid, rapid and slow freezing can be employed, depending on the species. Cassava and potato meristems survive direct immersion in liquid nitrogen (Bajaj, 1977a; Grout & Henshaw, 1978). However, Towill (1983), using potato meristems coming from *in vitro* cultured plantlets, obtained regrowth using slow freezing (0.2 to 0.3° C · min^{-1} to – 35° C). The type of development after thawing depends on the freezing method. Potato meristems show callusing after rapid freezing (Benson et al., 1989). On the contrary, direct regrowth is obtained after slow freezing. Thawing is usually rapid, by immersion of the material in a water bath or in sterile medium thermostated at 35–40° C. Recovery generally occurs directly on the standard medium.

3.2.1.5 Embryos

The main characteristic of this type of material is its size, which is generally large, by cryopreservation standards. The embryos often comprize differentiated tissues, depending on their developmental stage. Thus, embryos as young and as immature as possible (e.g. globular stage) will be preferentially used. For embryo cryopreservation, two different categories of material can be considered: zygotic embryos, which are harvested from *in vivo* material and placed into *in vitro* conditions only after cryopreservation, and somatic embryos which are already cultivated *in vitro*. The challenge is different for these two categories: for zygotic embryos, the whole structure has to be preserved in order to give rise to a whole plant, whereas with somatic embryos, only the proliferation capacities of the material must be preserved and not necessarily their structural integrity. For this latter group, standard cryopreservation techniques are used. An additional stage, prior to cryopreservation may be necessary, in order to produce a particular type of material, i.e. embryos at the right developmental stage. With oil palm, only shiny white, finger-like shaped embryos grouped into clumps are likely to withstand freezing. They are obtained after a two-month culture on a medium enriched with sucrose, as indicated earlier (Engelmann & Dereuddre, 1988a). After a culture for several days in presence of cryoprotective substances, the embryos are pretreated with cryoprotective compounds, 10% DMSO for *Citrus sinensis* somatic embryos (Marin & Duran-Vila, 1988), sucrose and DMSO for coffee somatic embryos (Bertrand-Desbrunais et al., 1988). The embryos are usually frozen in liquid medium. However, dry freezing (Withers, 1979) is employed with *Citrus sinensis* (Marin & Duran-Vila, 1988), oil palm (Engelmann et al., 1985) and cassava somatic embryos (Sudarmonowati & Henshaw, 1990). The freezing rates must be precisely determined, 0.5° C · min^{-1} to – 42° C for *Citrus*

sinensis somatic embryos (Marin & Duran-Vila, 1988), rapid freezing for rice pollen embryos (Bajaj, 1981). However, in the case of oil palm somatic embryos, a wide range of cooling rates (0.1 to 200° C · min^{-1}) can be employed (Engelmann & Dereuddre, 1988b). Thawing is usually rapid, with an exception for carrot and *Citrus sinensis* embryos which are slowly rewarmed at room temperature (Withers, 1979; Marin & Duran-Vila, 1988). There are different possibilities offered for regrowth: the embryos may be transferred directly onto standard medium, or media modified by transitory addition of growth regulators (Engelmann et al., 1985), or compounds with osmotical properties (Bertrand-Desbrunais et al., 1988) may be used.

In the case of zygotic embryos, which are excised from the seed and frozen immediately, the cryopreservation process is generally different. The cotyledons are oftenly removed and only embryonic axes are used. The partial dehydration usually provided by the contact with the cryoprotective solution, is obtained by placing the explants under the laminar flow and letting them dehydrate in the air current. The intensity of this dehydration is adapted to the desiccation tolerance/sensitivity of species. For example, *Hevea* freshly excised embryonic axes have a moisture content of 55% and 100% viability without any treatment, but do not tolerate freezing in liquid nitrogen. After 3 hours desiccation, the water content drops to 16%, the viability is 87% and the survival after freezing reaches 67% (Normah et al., 1986). Rapid dry freezing is usually employed, but controlled slow cooling (2° C · min^{-1}) proves to be successful with cassava embryonic axes (Marin et al., 1990). Thawing is usually performed by placing the cryotubes in the air current of a laminar flow cabinet. In the case of cassava zygotic embryos, the rewarming rate is slower, by firstly placing the cryotubes for one hour in a deep freezer at − 70° C and then at − 15° C before reaching room temperature (Marin et al., 1990). Regrowth generally takes place directly on the standard medium.

3.3 New cryopreservation techniques

The aim of these new freezing techniques is to look for simplifications to standard cryopreservation protocols.

3.3.1 Encapsulation/Dehydration

This technique is adapted from the medium term storage experiments carried out by Bapat et al. (1987, see 2.1.8). It has been developed by a french research team, using meristems of potato (Fabre & Dereuddre, 1990), grape (Plessis et al., 1991), pear (Scottez et al., 1991) and somatic embryos of carrot (Dereuddre et al., 1991). It is based on the fact that encapsulation protects the structure embedded and makes it resistant to treatments which otherwise would be lethal. The alginate beads containing the explants are cultivated for several hours/days in a liquid medium with a high sucrose level, then partially desiccated under the laminar flow and frozen either slowly or rapidly. After slow thawing, the beads are transferred to standard medium. Regrowth of the explants is satisfactory. In the case of potato, 26% of the frozen apices could develop into plants.

3.3.2 Vitrification

This technique was developed recently by various authors (Uragami et al., 1989; Langis et al., 1989; Langis & Steponkus, 1990; Sakai et al., 1990; Towill, 1990), using cell suspensions, protoplasts, somatic embryos and meristems of various species. In a vitrification process, the material is frozen ultrarapidly, in order that the water vitrifies, i.e. forms an amorphous glassy structure, thus avoiding the problems caused by ice formation inside the cells. In order to achieve vitrification, a rapid and very precisely timed pretreatment in the presence of very high cryoprotectant concentrations is needed. Dilution of the cryoprotective medium, after thawing, is also very precise. It seems that, at least for cell suspensions, the complexity of the freezing phase in the standard procedure is transferred to the pretreatment phase, making this technique far from being extensively usable in the near future.

3.3.3 Use of a domestic freezer

This technique was developed recently by Tessereau et al. (1990), using coffee embryogenic cell suspensions and somatic embryos. After a standard

pretreatment with high sucrose concentrations, the cryotubes containing the suspension are placed in a commercial freezer at −20° C for 24 hours, then immersed in liquid nitrogen. A 50% viability rate (measured by TTC reduction) could be obtained immediately after thawing. After 42 days in culture, the performance of the cryopreserved line, in terms of the final cell density, the productivity of embryos and the percentage of embryos at the torpedo stage were similar to unfrozen control.

3.3.4 Potential use for these new techniques

These new techniques, vitrification, encapsulation/dehydration, use of domestic freezer, will certainly not replace the conventional processes but they present two main interests. Firstly, they may offer additional possibilities for plant materials which are recalcitrant to conventional freezing procedures. Secondly, they may offer the possibility of avoiding the use of a programmable freezer, which would simplify the process, thus making it less costly and facilitating its utilization.

3.4 Trueness to type, storage duration

The possible variations of the material due to cryopreservation have been principally checked on the production of particular compounds by cell strains (Seitz, 1987). Until now, no modifications of the properties of stored material have been observed after thawing (Table 2). For organized structures, plants obtained from frozen meristems (Bajaj, 1983b, 1985; Kartha et al., 1980) or embryos (Engelmann, 1990a) of several species were phenotypically normal. Only limited analysis of the molecular stability of cryopreserved plant material has been currently carried out. RFLP analysis performed with shoot-tips of *Solanum tuberosum* showed no changes after cryopreservation (Harding, 1990). Similar results were observed with transformed roots of *Beta vulgaris* and *Nicotiana rustica* checked for their T-DNA structure (Benson & Hamill, 1991). However, in a recent work with *Triticum aestivum,* Kendall et al. (1990) could select freezing tolerant calluses by repeated exposures to liquid nitrogen. The cryoselected calluses regenerated plants with enhanced cold hardiness. The seed progeny of some of the lines tested exhibited significantly enhanced tolerance to −12° C, thus indicating that cryoselection appears to involve, at least in part, selection for genetic rather than epigenetic variants. The implications of this work may be of great importance in the case of tropical species, which are cold sensitive. Indeed, the possibility of producing plants with increased cold resistance is very interesting.

Regarding storage duration, experience is very limited with plant material (see Table 2). Indeed, the maximal storage duration that has been tested is 4 years, in the case of cassava and potato meristems (Bajaj, 1985). Until now, all storage experiments led to the production of true-to-type material.

Table 2. Maximal storage duration in liquid nitrogen experimented for plant species cultured *in vitro* and stability of regenerated plants

Species	Material	Storage duration (years)	Reference
Digitalis lanata	cell suspension	3	Diettrich et al., 1985
Fragaria ananassa	meristems	2 (normal plants)	Kartha et al., 1980
Arachis hypogea	meristems	2 (normal plants + seeds)	Bajaj, 1983d
Manihot esculenta	meristems	4	Bajaj, 1985
Solanum tuberosum	meristems	4	Bajaj, 1985
Elaeis guineensis	somatic embryos	1.5 (normal plants)	Engelmann et al., 1986

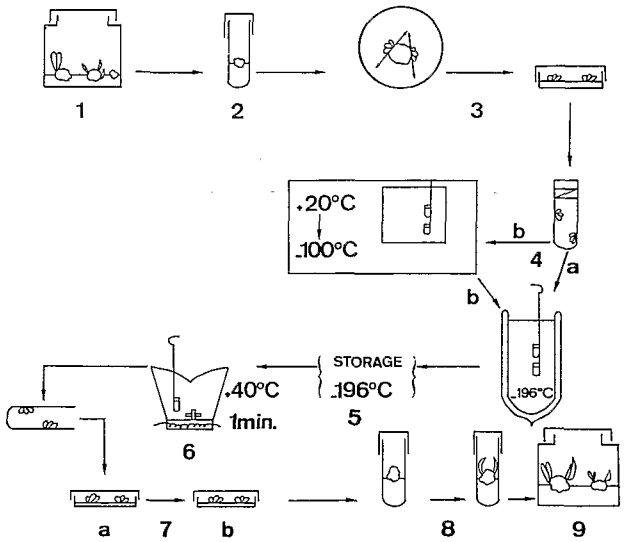

Fig. 1. Schematical representation of the oil palm somatic embryo cryopreservation process (After Engelmann, 1986).

1. Starting material.
2. Production of embryoid clumps for freezing: 2 months on sucrose 0.3 M.
3. Dissection of clumps and 7-day pretreatment on sucrose 0.75 M.
4. Freezing: a) rapid b) programmed.
5. Storage in liquid nitrogen (− 196° C).
6. Thawing: 1 min at 40° C.
7. Post-treatment:
   a) 1 wk on sucrose 0.3 M + 2.4-D 10^{-6} M
   b) 2 wks on sucrose 0.1 M + 2,4-D 10^{-6} M.
8. Successive transfers on multiplication medium: resumption of multiplication.
9. Transfer to jars to allow cultures to expend and produce ramets from frozen embryoid clumps.

3.5 Present development of cryopreservation: example of oil palm somatic embryos

Although resistance to freezing to the temperature of liquid nitrogen has been demonstrated for a large number of species, the current application of cryopreservation as a routine technique is exceptional. The first example concerns oil palm (Engelmann, 1986).

The oil palm vegetative propagation process set up by ORSTOM and IRHO (Institut de Recherche pour les Huiles et Oléagineux) uses somatic embryogenesis (Pannetier et al., 1981). This process is now applied on an industrial scale in 5 laboratories in France, Ivory Coast, Malaysia and Indonesia.

Research to set up a cryopreservation procedure started in France in 1982, in order to resolve the following problems:

– on the one hand, the risks of obtaining abnormal material which increases with *in vitro* culture duration, as it was shown in the case of oil palm (Corley et al., 1986). Storing the embryos as early as possible after they have been obtained should increase our chances of storing true-to type material.

– on the other hand, the continuous production of new clones induces laboratory management problems. Cryopreservation allows to store the clones which are not used for commercial production, thus reducing the quantity of material which has to be regularly subcultured.

The cryopreservation process which has been set up (Engelmann, 1986) is presented in Fig. 1. The following conditions have been defined (Numbers in parentheses in the following text refer to Fig. 1):

– Choice of material: only young embryos, shiny white and finger-like, often grouped into clumps, are likely to withstand freezing. They are obtained in sufficient numbers after two months of culture on medium enriched with sucrose (1–2).

– Pretreatment: the clumps of embryos are placed for 7 days on a medium containing 0.75 M sucrose. Their water content decreases from 80% to around 60% (3).

– Freezing: the clumps are placed in sterile cryotubes and frozen rapidly by direct immersion in liquid nitrogen ($-200° C \cdot min^{-1}$) (4a). A two-step freezing can be carried out using a programmable freezing apparatus: the cryotubes are frozen from + 20° C to − 100° C at a rate which can vary from 5 to $40° C \cdot min^{-1}$, then plunged in liquid nitrogen (4b).

– Thawing: the cryotubes are plunged in a water-bath thermostated at + 40° C for 1 minute (6).

– Post-treatment: the embryo clumps are cultured for 3 weeks on media supplemented with 2,4-D and containing progressively less sucrose (7a–b). Afterwards, they are transferred on the standard medium devoid of growth regulators (8–9).

The process described above has been successfully applied to 27 different clones, with an average recovery rate of 12.5% (Engelmann, 1990a). It has been checked with 2 clones that the extension of

the storage duration in liquid nitrogen to 12 and 15 months respectively did not modify the recovery rate. Finally, plantlets from two cryopreserved clones have been produced and planted in the field at the IRHO La Mé research station in Ivory Coast. No difference was observed when compared with non frozen controls.

This technique is currently being developed in the laboratories producing oil palm embryos through the ORSTOM-IRHO system. The first results show that it could be successfully applied. Slight adaptations of the technique to the different plant materials cultured in these laboratories may be necessary, in order to improve the present results.

4. Conclusion

In conclusion, tissue culture techniques, together with cryopreservation, are of great interest for the medium and long-term conservation of plant germplasm, particularly that of tropical species. However, before routine procedures can be foreseen using *in vitro* techniques for germplasm preservation, various problems have to be faced. The germplasm has to be evaluated in order to store a representative sample of the variability of the species. Moreover, a minimal knowledge of the biology and physiology of the species is needed. *In vitro* culture conditions have to be determined for the species which has to be conserved. Finally, trials must be carried out in order to determine precisely the conditions for slow growth storage as well as for cryopreservation. Practical problems exist as well, particularly in the developing countries: existence of minimal tissue culture facilities, lack of funds, of trained personnel, of a regular and reliable supply of liquid nitrogen.

If routine techniques can be set up easily for medium-term storage of *in vitro* plant collections by slow growth, this is not the case for cryopreservation. Resistance to the temperature of liquid nitrogen has been proved for a large number of species. However, the use of cryopreservation is limited to small laboratory collections and its use on a large scale is currently exceptional. Indeed, the successful, routine freezing of plant material, which implies the setting up of very precise conditions, often requires extensive research using sophisticated equipment. In this context, the search for less sophisticated freezing techniques, such as encapsulation/dehydration, vitrification or the use of a domestic freezer is of great interest and may be helpful in certain cases. However, the use of conventional freezing techniques may still remain obligatory in the majority of the cases.

Over the last few years, national and international bodies, public research institutes and private firms have shown increasing interest in germplasm storage and cryopreservation. This encourages us to feel optimistic about the development of routine techniques for the safe storage of tropical germplasm.

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